Influence of Nutrients involved in One-Carbon Metabolism on DNA Methylation in Adults

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Title: Influence of Nutrients involved in One-Carbon Metabolism on DNA Methylation in Adults - A Systematic Review and Meta-Analysis

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Abbreviations used: CI, confidence interval; CVD, cardiovascular disease; DMP, differentially methylated position; DMR, differentially methylated region; LC-MS, liquid chromatography-tandem mass spectrometry; LINE-1, long interspersed nuclear elements; MTHFR, methylenetetrahydrofolate reductase; RBC, red blood cell; RCT, randomized controlled trial; SAM, S-adenosylmethionine; SD, standard deviation; SEM, standard error of mean; TSS, transcription start site; UTR, untranslated region.

ABSTRACT

Context: Aberrant DNA methylation is linked to various diseases. The supply of methyl groups for methylation reactions is mediated via S-adenosylmethionine which depends on the availability of folate and related B-vitamins.

Objectives: To investigate the influence of key nutrients involved in one-carbon metabolism on DNA methylation in adults.

Data sources: Systematic literature searches were conducted in the Cochrane library, Medline, Embase, CINAHL Plus, Scopus and Web of Science databases. Studies that met the inclusion criteria and were published in English were included.

Data extraction: The first author, study design, sample size, population characteristics, type of intervention and duration, tissue type or cells analyzed, molecular techniques and DNA methylation outcomes.

Data synthesis: A meta-analysis of RCTs was conducted to investigate the effect of one-carbon metabolism nutrients on global DNA methylation. Functional analysis and visualization was performed using BioVenn software.

Results: From a total of 2620 papers screened by title, 53 studies met the inclusion criteria. Qualitative analysis indicates significant associations between one-carbon metabolism nutrients and DNA methylation. In meta-analysis of RCTs stratified by method of laboratory analysis, supplementation with folic acid alone or in combination with vitamin B-12 significantly increased global DNA methylation in studies employing LC-MS, which had markedly lower heterogeneity (n = 3, $Z = 3.31$, $P = 0.0009$; $I^2 = 0\%$) in comparison to other methods. Functional analysis highlighted a subset of 12 differentially methylated regions that were significantly related to both folate and vitamin B-12 biomarkers.

Conclusions: This study supports significant associations between one-carbon metabolism nutrients and DNA methylation. However, standardization of DNA methylation techniques is recommended to reduce heterogeneity and facilitate comparison across studies.

Systematic Review registration: PROSPERO registration number: CRD42018091898.

Key words: One-carbon metabolism nutrients, DNA methylation, B-vitamins, one-carbon metabolism, systematic review, meta-analysis

INTRODUCTION

DNA methylation is the most stable epigenetic mechanism in mammals. It is important in the regulation of gene expression and maintaining genome stability both locally and at the global level [1,2]. Changes in methylation occur in utero or in early life and are subject to age-related changes during an organism's lifetime [3–6]. This systematic review focuses on methylation changes during adult life. Aberrant DNA methylation in adults has been linked to aging and implicated in many diseases including cancer and cardiovascular disease [1,2,4]. Understanding the role of B-vitamins in regulating DNA methylation in aging and their roles in disease pathophysiology is essential in both the diagnosis and treatment of many diseases [7].

DNA methylation has been shown to be responsive to environmental shifts such as changes in diet or nutritional status [8,9]. Through the interaction with nutrients involved in one-carbon metabolism, methylation of specific genes can be modified, influencing gene expression and phenotypes [10–12]. Additionally, nutritional status can interact with specific genetic variants of key genes in one-carbon metabolism to modulate health offering a unique opportunity for dietary based interventions that target diseases linked to altered DNA methylation [13,14].

One-carbon metabolism is one of the main metabolic networks by which nutrients interact biologically to modulate DNA methylation. Nutrients involved in one-carbon metabolism include folate, vitamin B-12, vitamin B-6, riboflavin (vitamin B-2), choline, betaine, methionine and homocysteine [15]. Folate and related B-vitamins provide the substrates and cofactors to ensure the efficient functioning of one-carbon metabolism [16–18]. Of particular note, the folate and methionine pathways in one-carbon metabolism generate S-adenosylmethionine (SAM), the universal methyl donor, required for numerous biological reactions including DNA, RNA and histone methylation.

Currently, evidence for the role of specific nutrients within the network on DNA methylation is conflicting. In several studies, intervention with B-vitamins, mainly folic acid, led to alterations in global, gene-specific or CpG site-specific DNA methylation [19–21], however conversely, other studies report no changes in methylation in response to folic acid or B-vitamin supplementation [22–24]. Furthermore, very little is known about doses, dietary exposure levels or extent of depletion necessary to elicit these epigenetic changes. Additionally, conditions such as life stage or health status at which one-carbon metabolism related nutrients have the largest modulatory effects on DNA methylation are currently not fully understood. There is therefore a need to systematically analyze and evaluate the current evidence for the influence of relevant nutrients involved in one-carbon metabolism on DNA methylation.

The aim of this study was to conduct a systematic review to investigate the influence of nutrients involved in one-carbon metabolism on DNA methylation in adult populations. In addition, a meta-analysis of RCTs was conducted to examine the effects of supplementation with relevant nutrients on global DNA methylation.

METHODS

This systematic review was conducted according to PRISMA guidelines (PRISMA checklist provided in **Supplementary Table S1)** and a registered protocol (PROSPERO 2018, CRD42018091898). Screening of eligible studies, full-text assessment, data extraction and quality assessment of studies was independently carried out by two authors, discrepancies were discussed and resolved by consensus and where necessary moderated by a third reviewer. Studies were selected in accordance with the PICOS (population, intervention, comparison, outcome, and study design) criteria shown in **Table 1**.

Search Strategy and Study Selection

Systematic literature searches were conducted in the Cochrane library, Medline (Ovid), Embase, CINAHL Plus, Scopus and Web of Science databases without any language restrictions in March 2019 (detailed search strategy provided in **Supplementary Table S2**). The full search strategy for all the searches combined terms related to one-carbon metabolism nutrients or synonyms (e.g. folate, vitamin B-12, riboflavin and vitamin B-6), DNA methylation (e.g. global, gene-specific, genome-wide methylation) and homocysteine are presented in **Supplementary Table S2**. Medical subject headings and key word searches were conducted in Embase, Medline, CINAHL Plus and Cochrane databases while searches in Scopus and Web of Science were carried out using only key word searches.

Following removal of duplicates, the titles and abstracts of studies retrieved from the literature search were screened for potentially eligible studies. Full text articles of potentially relevant articles were further reviewed using a pre-designed in/out form which included questions to assess each study's relevance for the review. Studies were considered eligible if they were original peer-reviewed full-text articles published in English and included all the defined outcomes.

Inclusion and Exclusion Criteria

Studies conducted in adult humans investigating all of the following: 1) DNA methylation (global, gene-specific and genome-wide methylation), 2) nutrients involved in one-carbon metabolism and 3) circulating homocysteine levels (potential biomarker of one-carbon metabolism) were included in the current review. Studies involving 1) pregnant women and children, 2) *in-vitro* studies using human or animal cell lines and 3) studies conducted in animals were excluded from the analysis.

Data Extraction, Synthesis and Analysis

Data extraction was carried out using a predesigned data collection sheet to extract relevant information from the selected studies. Information extracted included the name of

first author, study design, sample size, population characteristics, type of intervention and duration (intervention studies and randomized controlled trials), type of tissues or cells analyzed, molecular techniques and outcomes related to DNA methylation.

A narrative synthesis using descriptive statistics such as frequencies and percentages are presented for all studies included. The effects of supplementation with nutrients involved in one-carbon metabolism on DNA methylation are reported for RCTs and intervention studies. A meta-analysis examining the effect of supplementation with nutrients involved in one-carbon metabolism on global DNA methylation is included for RCT studies. Associations between one-carbon metabolism nutrients and DNA methylation are reported for observational studies. Owing to the considerable heterogeneity in study aims, designs and evaluated outcomes in the observational and intervention studies included, no quantitative analysis could be carried out for these type of studies.

Assessment of Risk of Bias

Risk of bias of RCTs and intervention studies was assessed using the following key criteria: random sequence generation, allocation concealment, blinding of participants and outcome, incomplete outcome data, selective reporting and other sources of bias in accordance to the Cochrane Risk of Bias Assessment tool [25]. The risk of bias in each study was classified as low risk, high risk or unclear risk (either a lack of information or uncertainty over potential bias). Risk of bias of observational studies were assessed for key criteria: selection, comparability and outcome using the Newcastle-Ottawa scale[26].

Quality of Reporting Studies

Quality of reporting the studies included in the review was assessed using the STROBE (STrengthening the Reporting of OBservational studies in Epidemiology) checklist [27] for observational studies, the CONSORT (Consolidated Standards of Reporting Trials) checklist [28] for RCTs and a modification of the TREND statement [29] for intervention studies

without randomization. All questions on the appropriate checklists were considered for the studies included. The final score for each study was based on adherence to appropriate checklist criteria. A percentage score was calculated as the number of checklist criteria adhered to divided by the total number of questions on the checklist.

Meta-Analysis of the Effects of Supplementation with Nutrients involved One-Carbon Metabolism on Global DNA Methylation

Meta-analysis of RCTs included in the review was conducted to examine the effects of supplementation with nutrients involved in one-carbon metabolism on global DNA methylation. No quantitative analysis could be carried out for the RCTs focusing on gene-specific methylation owing to the diverse range of candidate loci examined and resulting paucity of data for each target. Similarly, only 1 genome-wide methylation study was returned from the search preventing its inclusion in the meta-analysis. The remaining RCTs investigating global methylation were considered for inclusion in the meta-analysis only if they included a placebo or control group.

Data synthesis for meta-analysis was conducted using the standardized EURECA guidelines [30]. In cases where 2 publications reported data from the same study, they were linked and treated as one "main" intervention study. With the use of this approach, Pufulete *et al*[19] and Al-Ghnaniem Abbadi *et al.*[22] were treated as one study and the global methylation results reported in Al-Ghnaniem Abbadi were excluded from the meta-analysis. Further LINE-1 methylation data from Obeid *et al.* [20] and Pusceddu *et al.*[31] were from the same study and hence the data reported in Obeid *et al*.[20] were used in the meta-analysis. Where studies used more than one intervention strategy with one common placebo group[32], each intervention arm and placebo group were treated as an independent study in the meta-analysis. Further, in studies where DNA methylation was measured at two different time

points post-supplementation [23] or in different tissues [19] in the same study, the results were treated as independent studies in the meta-analysis.

Statistical Analysis

Review Manager 5.3 software (Cochrane Collaboration, 2014) was used to perform the meta-analysis. Mean methylation values and corresponding standard deviations were extracted from the included studies. In studies where the measure of variance was reported as SEM or CI [19,23,24,33,34], the SD was estimated using Cochrane formulas [35]. The overall pooled effect (Z) was analyzed using the standardized mean difference and the random effects model. The random effects model estimates the between-study variance and uses this estimate to modify the weights assigned to individual studies when calculating the overall effect [36]. In order to establish if methylation is confounded by heterogeneity in one-carbon metabolism nutrient supplemented, DNA methylation technique or tissue analyzed, pre-specified subgroup and sensitivity analyses was carried out for each of those variables. Data are expressed as standardized mean difference (95% CI) and the overall effect Z (*P*-value).

Statistical heterogeneity was evaluated using chi square value, heterogeneity index (I^2) statistics and corresponding *P*-value. Heterogeneity thresholds were defined according to Cochrane guidelines, with I^2 between 0 - 40% indicative of low heterogeneity, I^2 between 30 - 60% representing moderate heterogeneity, I^2 between 50 - 90% representing substantial heterogeneity and I^2 between 75 - 100% indicating considerable heterogeneity [35]. Potential publication bias for each study included in the meta-analysis was assessed by visual inspection of funnel plots and Egger's regression test [37].

Functional Analysis of Epigenome-wide Methylation

Functional analysis was carried out using differentially methylated regions (DMRs) previously identified to be related to both serum folate and vitamin B-12 in epigenome-wide methylation studies [21]. The DMRs were identified using the DMRcate package available

200 through Bioconductor in R statistical environment. Overlapping DMRs associated with serum
201 folate or vitamin B-12 levels were visualized using BioVenn Software [38].

202 **RESULTS**

203 A total of 2620 records were identified through searches in 6 databases. After
204 screening and removal of duplicates, 127 records were assessed for eligibility and 59 records
205 subjected to full-text assessment. Six additional records which did not clearly report any
206 associations between nutrients involved in one-carbon metabolism and DNA methylation
207 were further excluded leaving 53 studies which are included in the qualitative analysis and 8
208 publications included in the meta-analysis. Study screening, eligibility and selection
209 processes are shown in **Figure 1**.

210 **Characteristics of Studies Included**

211 *Study design and background characteristics*

212 Summary and key findings of the studies included are provided in **Tables 2-6** [13,20–
213 24,31–34,39–81]**.** Overall, data from 9561 adults with ages ranging from 18-85 years were included
214 in this systematic review. Study participants were from 13 countries (USA, UK, Germany,
215 Italy, the Netherlands, Sweden, Australia, Malaysia, Poland, China, Chile, Korea and
216 Ireland). The majority (74.6%; n = 41) of studies involved both male and female participants.
217 RCTs and intervention studies without randomization constitute 29.1% (n = 16) and 18.2% (n
218 = 10) of studies reviewed respectively while 52.7% (n = 29) of the studies were observational
219 (cross-section, case-control and cohort). While 1 publication reported both RCT and cross-
220 sectional data [23], another publication reported data for both an intervention and RCT [40]. Of
221 the intervention studies included, 6 were depletion-repletion studies [40,46,49,50,53,54] and another
222 3 were supplementation studies without randomization [47,51,52]. Studies were conducted mainly
223 in healthy individuals (34.6%, n = 19), or those with cancer (27.3%, n = 15), CVD (9.1%, n =
224 5), elderly subjects (9.1%, n = 5) and other diseases or conditions (20.0%, n = 11).

One-carbon metabolism nutrients examined

The main one-carbon metabolism nutrient supplemented or examined in most studies (RCTs, intervention and observational) was folate (40.7%, n = 22), 18 studies (33.3%) examined both folate/folic acid and vitamin B-12 status, 13 studies (24.1%) examined a complex of B-vitamins and calcium and 1 study (1.9%) investigated methionine [76]. A large proportion of studies measured biomarkers (75.0%, n = 39), 9 studies (17.3%) reported both biomarker and dietary intake and 2 studies (3.8%) reported only dietary data. Of the 16 RCTs, 9 [19,22,24,32–34,41,42,45] supplemented with folic acid only (with doses ranging from 100µg/d to 1500µg/d). While 4 RCT studies intervened with a combination of folic acid and vitamin B-12 [21,23,32,44], another 4 RCT studies supplemented with folic acid and other B-vitamins [20,31,40,43]. Duration of RCTs ranged from 10-156 weeks.

Furthermore, intervention studies using the depletion-repletion study design, fed participants a folate-restricted diet (56µg-79.4µg/d) during the depletion stage and a folate treatment diet (111µg-516µg/d) during the folate repletion stage. Observational studies examined mainly circulating biomarker concentrations of folate and vitamin B-12 (51.7%, n = 15), or circulating biomarker concentrations of several one-carbon metabolism nutrients including folate, B-12, B-6, B-2, betaine, choline and methionine (34.5%, n = 10) and 4 studies (13.8%) examined only folate status.

DNA methylation Analysis

Studies focused on a range of genomic locations and DNA methylation was assessed in a variety of tissues using different methods. While 67.3% of studies (n = 37) examined global methylation, 20.0% (n = 11) measured gene-specific methylation, 1 study (1.8%) examined genome-wide methylation [21] while 6 studies (10.9%) examined both global and gene-specific methylation [20,22,52,59,60,68]. Methylation was examined mostly in blood (whole blood, leukocytes, monocytes and peripheral blood cells; 74.6%, n = 41), colorectal tissue

(20.0%, n = 11) or both blood and colon tissues (5.5%, n = 3). DNA methylation analyses were carried out using 16 different techniques, mainly pyrosequencing (n = 14), LC-MS techniques (n = 12), methyl acceptance assay (n = 12) and 6 studies used more than one method.

Effect of Supplementation with Nutrients Involved in One-Carbon Metabolism on DNA Methylation

The effect of supplementation with nutrients involved in one-carbon metabolism on DNA methylation was investigated in 16 studies using RCT study design[19–24,31–34,40–45] (**Table 2**). While the largest proportion of RCTs examined the effect of one-carbon metabolism nutrients on global methylation (68.8%, n = 11), 2 studies (12.5%) investigated gene-specific methylation [44,45], another 2 studies (12.5%) examined both global and gene-specific methylation (12.5%) and 1 study (6.3%) examined genome-wide methylation. While 61.5% (n = 8) of RCTs examining global DNA methylation observed no significant changes in methylation in response to supplementation [22–24,32,34,40,41,43], 38.5% (n = 5) observed significant increases in methylation [19,20,31,33,42]. Furthermore, RCTs investigating gene-specific methylation in colorectal adenoma patients and elderly subjects showed significant increases in colorectal tissue and blood DNA methylation at several loci including *ASPA*, *PDE4C, MGMT, MLH1, p14, p16* and *RASSF1A* [20,44] in response to nutrient supplementation; however no significant effects were observed for *ESR1, ITGA2B, MLH1* and *SFRP1* methylation [20,22,45].

In the single RCT investigating the effects of supplementation with folic acid and vitamin B-12 on epigenome-wide methylation in adult leukocyte samples [21], 6 significant differentially methylated regions (DMRs) between intervention and placebo groups were discovered. Intervention with folic acid and B-12 in this study increased DNA methylation for the majority of *HOX* genes while remaining stable or decreasing in the placebo group. In

addition to comparisons for DNA methylation changes between these two groups, the relationship between DNA methylation and serum folate was examined in a continuous manner, revealing that for 91% of the top 35 differentially methylated positions (DMPs), DNA methylation was positively correlated with levels of serum folate. Furthermore, 173 and 425 DMRs, were significantly associated (Benjamini-Hochberg adjusted p-value < 0.05) with serum folate and vitamin B-12 concentrations respectively in this study [21].

Global methylation was examined in 90% (n = 9) of studies using an intervention study design without randomization with 1 study [52] investigating both global and gene-specific methylation (**Table 2**)[40,46–51,53,54]. Although 3 (60.0%) intervention studies conducted in both pre and postmenopausal women report decreased global methylation during folate restriction [50,53,54], and 2 studies (40.0%) conducted in healthy premenopausal women observed no effect in response to depletion [40,46]. Conversely, in the intervention studies employing supplementation, 50.0% of these (n = 5) observed effects of supplementation on global methylation [46–50,54] and 50.0% (n = 5) did not observe any changes in methylation in both healthy populations or those with elevated homocysteine [40,48,51,53]. In the single intervention study investigating gene-specific methylation conducted in apparently healthy adults at increased risk of colorectal adenoma, there was no significant effect on methylation of 432 genes known to be abnormally methylated in human cancers [52].

Association between Nutrients involved in One-Carbon Metabolism and DNA Methylation

The majority of observational studies examined global methylation (56.7%, n =17), while 10 studies examined gene-specific methylation (33.3%) and three studies (10.0%) examined both (**Tables 3- 5**) [13,23,55–81]. Several observational studies indicate significant associations between nutrients and global methylation. While 9 studies (50.0%) direct associations [58,66,70,71,76,78,81,82], 3 studies (16.7%) involving cancer patients or healthy

300 participants observed inverse correlations [64,69,74] and 6 studies (33.3%) did not observe any
301 significant associations [23,59,60,62,65,73]. Two studies conducted in atherosclerosis patients and
302 older participants measuring global methylation using 2 different surrogate markers of global
303 methylation, observe positive associations between B-12 or B-6 status and Alu but not LINE-
304 1 methylation [63,67]. A further 9 observational studies (75.0%) report significant associations
305 between nutrients and methylation of specific gene loci with positive correlations observed
306 for *VDR, p73, MTHFR, CACNA1G* and *RUNX3* [55,72,75,77,79] but negative correlations for
307 *TNFA, MLH1, MGMT* and *ESR1* in cancer or obese patients [13,56,68]. Three studies (25.0%) did
308 not observe significant correlations between nutrient status and *ec-SOD, p66Shc* and *TERT*
309 methylation [59,61,80].

310 **Nutrients involved in One-Carbon Metabolism, Global Methylation and *MTHFR***
311 **C677T Genotype**

312 The *MTHFR* C677T polymorphism is a common polymorphism associated with
313 reduced activity of the MTHFR enzyme and thereby affecting folate availability in one-
314 carbon metabolism [83]. Sixteen studies examined the relations between nutrients involved in
315 one-carbon metabolism (mainly folate), global methylation and the *MTHFR* C677T genotype.
316 Low folate status was associated with lower methylation in *MTHFR* 677TT genotype
317 participants compared to CC subjects [58,66,82,84]. Furthermore, decreases in methylation were
318 observed in participants with the *MTHFR* 677TT genotype in response to folate
319 supplementation in healthy young women [41,46,54]. On the contrary, 6 studies found no
320 significant effect or association between folate status and DNA methylation in individuals
321 stratified by the *MTHFR* C677T genotype [24,40,44,48,60,62]. Although stratification by *MTHFR*
322 C677T genotype revealed 5 positions with differential methylation in response to B-vitamin
323 supplementation, the power of the subgroup analysis was limited owing to low numbers and
324 results should be interpreted with caution [21].

Risk of Bias and Quality of Reporting Studies

The quality of evidence presented in the included studies was rated as moderate with an average score of 65.2% on the quality assessment scales. Overall, RCT studies showed low risk of bias for random sequence generation, (93.8%), allocation concealment (75.0%), blinding of participants and personnel (75.0%), blinding of outcome assessment (37.5%), incomplete outcome data (43.8%) and selective reporting (37.5%) bias domains while all studies showed unclear risk of bias in other bias owing to lack of sufficient information to assess whether an important risk of bias exists. The majority of intervention studies showed an unclear risk of bias in all the domains owing to insufficient information provided to permit judgement (**Supplementary Table S3**). Observational studies showed high comparability and reporting of outcomes but were rated lower on the selection scale using the Newcastle-Ottawa scale (**Supplementary Table S4)**. Although the quality of reporting studies was rated as good, several of the studies showed an unclear risk of bias highlighting the need for high quality studies with DNA methylation as the primary outcome.

Meta-Analysis on the Effect of Supplementation with Nutrients Involved in One-Carbon metabolism on Global DNA Methylation

The meta-analysis examined the effect of supplementation with one-carbon metabolism nutrients on global DNA methylation. It included data pooled from 918 individuals across 8 RCT studies. Firstly, 9 publications were considered for inclusion in the meta-analysis. Of these, 1 study [43] was excluded through lack of numerical data and although attempts to contact the author was made, the data could not be obtained. A study reported post-supplementation data at 2 time points [23], 1 study reported methylation data for both leukocytes and colon tissue [19] and another study [32] reported the effect of folic acid and a combination of folic acid and vitamin B-12 separately on global methylation. Although median values of methylation were reported in Kim *et al.* [42], the corresponding dispersion

was not available. For the purposes of the meta-analysis, the SD value was extrapolated from Fenech *et al.* [23], as similar methods were used for examining DNA methylation and methylation values were expressed in the same units.

Meta-analyses using the random effects model (**Figure 2**)[19,20,23,24,32–34,42] showed no significant overall effect of one-carbon metabolism nutrients on global DNA methylation (Z = 0.03, P = 0.98; I^2 = 64%, P = 0.002). Pre-specified subgroup analyses of methylation in blood and colorectal tissue also indicated no effect of nutrient supplementation on global methylation in either blood (Z = 0.28, P = 0.78) or colon (Z = 0.60, P = 0.55). Substantial heterogeneity was observed in blood (I^2 = 71%, P = 0.002, n = 7) and non-significant moderate heterogeneity was observed for colorectal tissue (I^2 = 48%, P = 0.13, n = 4) in subgroup analyses.

Further pre-specified subgroup analysis focusing on the assay used to quantify global DNA methylation was carried out to attempt to explain the substantial heterogeneity among studies (**Figure 3**) [20,23,24,32,34,39,42]. Studies that assessed global DNA methylation by LC-MS techniques showed that B-vitamin supplementation significantly increased global DNA methylation (Z = 3.31, P = 0.0009). This finding was in contrast to the results of the individual studies, which did not find a significant effect of B-vitamin supplementation on DNA methylation. There was no detectable effect in studies using pyrosequencing (Z = 0.40, P = 0.69) and methyl acceptance assay (Z = 0.18, P = 0.85). No heterogeneity was observed for studies employing LC-MS techniques (I^2 = 0%, P = 0.60, n = 3) compared to pyrosequencing (I^2 = 76%, P = 0.04, n = 2) and methyl acceptance assay (I^2 = 64%, P = 0.03, n = 5). When analyses were focused on intervention with either folic acid or combination of B-vitamins (**Supplementary Figure S1**) [20,23,24,32–34,39,42], subgroup analysis indicated no significant effect on DNA methylation owing to supplementation with folic acid only (Z = 0.52, P = 0.60) or folic acid in combination with B-12 and B-6 (Z = 0.52, P = 0.61).

Substantial heterogeneity was observed in the subgroup supplemented with folic acid only (I^2 = 71.0%, P = 0.002, n = 7) and B-vitamin combination (I^2 = 56%, P = 0.08, n = 4).

Publication Bias

Publication bias assessment by visual inspection of the funnel plot did not indicate any substantial asymmetry and this was confirmed by a non-significant Egger's regression test (P = 0.152).

Sensitivity Analysis

A sensitivity analysis was performed by omitting one study at a time and assessing the pooled effect (Z) for the remaining studies. The pooled overall effect was consistent and within an acceptable range of 0.04 (P = 0.97) to 0.57 (P = 0.57). These findings indicate that the overall effect and heterogeneity are not significantly influenced by any particular study included in the meta-analysis.

Functional Analysis of Epigenome-Wide Methylation

Further functional enrichment analysis was carried out on 173 and 425 DMRs which were shown to be significantly related to serum folate and vitamin B-12 status respectively (BH-adjusted p-value < 0.05) in the single epigenome-wide methylation RCT [21]. The present analysis highlighted a subset of 12 DMRs (based on the exact genomic coordinates) which were significantly associated with both serum folate and vitamin B-12 status (**Figure 4a**). These are referred to as overlapping DMRs. The list of genes mapped to these DMRs are listed in (**Supplementary Table S5**). The overlapping DMRs were located in the first exon, gene body, TSS200, TSS1500, 3′UTR and 5′UTR (**Figure 4b**).

DISCUSSION

This study encompasses qualitative and quantitative data to provide comprehensive evidence for the significant relationship between nutrients involved in one-carbon metabolism and DNA methylation across a range of health outcomes. The results from this systematic review indicate a significant role for specific nutrients in modulating both global and gene-specific methylation in a wide spectrum of diseases. Additionally, meta-analysis of a predefined subset of RCTs stratified by analytical method showed a significant increase in global methylation in response to B-vitamin supplementation for studies employing sensitive LC-MS techniques (n = 3). This functional relationship between one-carbon metabolism nutrients and global methylation has not been previously estimated in meta-analysis of randomized trials.

While limited by the small number of RCTs in the meta-analysis of the LC-MS subgroup, a small but significant increase in global DNA methylation following supplementation with folic acid alone or in combination with vitamin B-12 was detected in comparison to studies using LINE-1 pyrosequencing or the methyl acceptance assay. LC-MS is an extremely sensitive quantitative measure of total cellular 5-methyl cytosine [85,86] with guidelines [87,88] published on standardization and validation of methods by both the Food and Drug Administration (FDA) and European Medicines Agency (EMA). It is perhaps not surprising, therefore, that no heterogeneity was observed in the LC-MS subgroup analysis, which facilitated detection of the effect of B-vitamin supplementation on DNA methylation. Pyrosequencing of LINE-1 is also considered to be a very sensitive method and a multicenter benchmarking study evaluating DNA methylation techniques demonstrated that pyrosequencing of repetitive elements gave rise to highly reproducible results [89]. However, in the current studies reviewed, heterogeneity may have arisen through the use of non-standardized protocols in various laboratories, resulting in analysis of varying regions of the

LINE-1 locus or assays using a varied number of CpG sites and thereby preventing detection of a significant effect. Although providing a reasonable estimate of global methylation, the methyl acceptance assay is a semi-quantitative assay, confounded by suboptimal enzyme activity and stability of SAM, resulting in large assay variability.

Furthermore, it is established that DNA methylation is highly tissue-specific and variability in tissues analyzed could mask potential associations between specific nutrients and DNA methylation [90]. Each of the studies in the LC-MS subgroup analyzed methylation in a single tissue type, i.e. blood, while LINE-1 pyrosequencing and methyl acceptance assay studies were conducted using a mixture of both blood and colorectal tissue further confounding the meta-analysis of these groups. LINE-1 pyrosequencing is often the preferred method over LC-MS for global methylation as the necessary expertise and equipment for LC-MS are not as widely available [91]. In order to enable a meaningful comparison of global methylation between studies using LINE-1 pyrosequencing, there is a need for researchers in the field to adopt a more standardized approach. Given the already proven reproducibility of the assay employed in laboratories of the BLUEPRINT consortium [89], a reasonable recommendation would therefore be the widespread adoption of this method.

Assessment of DNA methylation in the studies reviewed here covered a wide range of genomic regions using 16 different techniques, introducing substantial variability and leading to confounding of outcome measurements and thereby posing significant challenges for comparability. It is therefore perhaps not surprising that the current meta-analysis did not detect an overall effect of supplementation with one-carbon metabolism nutrients on global methylation. In contrast to the current findings, a recent meta-analysis [92] reported increased global DNA methylation in response to folic acid in colorectal mucosa but not blood. This result was, however, largely driven by a single study [93] that was shown by the authors to display publication bias. In agreement with the current investigation, reanalysis of the data

449 excluding this study did not detect a significant effect of folic acid on global methylation [92].
450 The study by Cravo *et al*. [93] did not in fact meet the strict inclusion criteria for the current
451 systematic review and meta-analysis. These discrepancies in findings from both studies are
452 explained by the limited number of RCTs which met the inclusion criteria for meta-analysis.
453 This therefore highlights the urgent need for further high quality, robustly designed RCT
454 studies of B-vitamin supplementation to identify epigenetic modification in response to B-
455 vitamin supplementation.

456 Folic acid was the main nutrient used for supplementation in the majority of the RCTs
457 included in the meta-analysis. It was also the main B-vitamin investigated in one-third of
458 intervention and observational studies. By solely focusing on folate, interventions may only
459 partly modify the dietary factors related to one-carbon metabolism that influence DNA
460 methylation as the availability of folate depends on other B-vitamins [24,94]. Furthermore, the
461 interactions between nutrients involved in one-carbon metabolism when supplemented in
462 combination is complex, currently not fully understood and could influence DNA
463 methylation through different mechanisms [94]. For example, in an RCT of long-term
464 supplementation with folic acid and vitamin B-12 in elderly subjects, methylation of 425
465 DMRs is significantly associated with serum vitamin B-12 concentrations, whereas only 173
466 DMRs are associated with serum folate status [21]. Novel functional analysis presented here
467 highlights a subset of only 12 DMRs significantly related to both serum folate and vitamin B-
468 12 status. The genes mapped to these DMRs will be a valuable resource for future studies
469 investigating the combined effects of B-vitamin supplementation on DNA methylation and
470 may provide future targets for epigenetic therapies. Similarly, the effect of one-carbon
471 metabolism nutrients on DNA methylation is influenced by specific gene mutations in the
472 one-carbon pathway and polymorphisms (including the *MTHFR* C677T polymorphism) that
473 affect availability of methyl groups for methylation reactions in one-carbon metabolism [83].

More comprehensive studies are required to examine the complex interaction between polymorphisms in one-carbon metabolism, B-vitamins and other related nutrients in relation to DNA methylation.

Strengths of this current study include the use of mixed qualitative and quantitative approaches to provide comprehensive evidence of the relations between nutrients involved in one-carbon metabolism and DNA methylation. Additionally, the strength of the meta-analysis was that only RCTs with the most robust design (i.e. RCTs with a parallel placebo or control group) were included. A potential limitation of the present study is that a meaningful quantitative pooling of data could only be performed for a small subset of RCTs owing to substantial heterogeneity in study aims, designs, population and health status, DNA methylation analysis techniques and tissues analyzed. Further, while most studies investigating gene-specific methylation and one-carbon metabolism nutrients followed a candidate gene approach, providing a valuable starting point for future investigations, there is the possibility that other loci, yet to be identified, which may also be influenced by one-carbon metabolism nutrients could have been overlooked.

CONCLUSION

In conclusion, the present systematic review supports a functional relationship between specific nutrients involved in one-carbon metabolism and DNA methylation. Meta-analysis, of the limited evidence currently available from RCTs, shows that supplementation with folic acid alone or in combination with vitamin B-12 resulted in an increase in global DNA methylation. The results of this study provide a foundation for further work, and highlight the need for future studies investigating the role of B-vitamins in epigenetic modifications associated with disease.

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DLM, CFH and MW designed the research; SDA, SR, JD conducted the research, SDA performed the statistical analysis; SDA, DEK and S-JT performed the functional analysis; SDA, CFH, MW and DLM wrote the article; HM, JJS and CPW carried out critical revision for important intellectual content. DLM had primary responsibility for the final content. All authors read and approved the final version of the manuscript.

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Supporting information: The following supporting information is available through the online version of this article at the publisher's website:

Figure S1: Meta-analysis of the effect of supplementation with nutrients involved in one-carbon metabolism on global DNA methylation sub-grouped by one-carbon metabolism nutrients.

Table S1: PRISMA checklist

Table S2: Systematic search strategy

Supplementary Table S3: Risk of bias assessment to randomized controlled trials and intervention studies investigating the effect nutrients involved in one-carbon metabolism on DNA methylation.

Supplementary Table S4: Risk of bias assessment of observational studies association between nutrients involved in one-carbon metabolism and DNA methylation

523 **Table S5:** Functional analysis of overlapping differentially methylated regions (DMRs)
524 related to both serum folate and vitamin B12 levels in epigenome-wide methylation analysis
525 **Table S6:** Full list of genes investigated in gene-specific methylation studies

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FIGURE LEGENDS

FIGURE 1. Flow diagram of study selection for systematic review and meta-analysis. [1]One publication reported data from both a cross-sectional study and RCT, another publication reported both RCT and intervention data.

FIGURE 2. Random-effects meta-analysis of the effect of supplementation with nutrients involved in one-carbon metabolism on global DNA methylation sub-grouped by tissues analyzed. The horizontal lines running through each square represent the 95% CI for each study. The diamonds indicate pooled effect and 95% CI for each subgroup and the overall effect (Z). Chi^2, chi-squared test assesses whether observed differences in results are compatible with chance alone; I^2, heterogeneity index (0–100%).

FIGURE 3. Random-effects meta-analysis of the effect of supplementation with one-carbon metabolism nutrients on global DNA methylation sub-grouped by methylation techniques. The horizontal lines running through each square represent the 95% CI for each study. The diamonds indicate pooled effect and 95% CI for each subgroup and the overall effect (Z). Chi^2, chi-squared test assesses whether observed differences in results are compatible with chance alone; I^2, heterogeneity index (0–100%).

FIGURE 4. Functional analysis of overlapping DMRs. a) A total of 12 DMRs were significantly associated with both folate and vitamin B-12 status in epigenome-wide studies. These DMRs are referred to as overlapping DMRs. b) Genomic locations of overlapping DMRs. TSS200, 200 base pairs around the transcription start site; TSS1500, 1500 base pairs around the transcription start site; 3′ UTR, 3′ untranslated region; 5′ UTR, 5′ untranslated region.

TABLE LEGENDS

TABLE 2. Randomized controlled trials investigating the effect of one-carbon metabolism related nutrient supplementation on DNA methylation (n 16). [a]Full name of genes provided in Supplementary Table S6. **Abbreviations:** GC-MS, gas chromatography-mass spectrometry, LC-MS, liquid chromatography-tandem mass spectrometry

TABLE 3. Intervention studies investigating the effect of nutrients involved in one-carbon metabolism on DNA methylation (n 10). **Abbreviations:** LC/ESI-MS, liquid chromatography electrospray ionization mass spectrometry; LC-MS, liquid chromatography-tandem mass spectrometry, LUMA, luminometric assay; PBMC, peripheral blood mononuclear cells; MTHF, methyltetrahydrofolate.

TABLE 4. Cross-sectional studies investigating the association between nutrients involved in one-carbon metabolism and DNA methylation (n 15). [a]Full name of genes provided in Supplementary Table S6. **Abbreviations:** LC-MS, liquid chromatography-tandem mass spectrometry; LUMA, Luminometric methylation assay, PBMC - peripheral blood mononuclear cells.

TABLE 5. Case-control studies investigating the association between nutrients involved in one-carbon metabolism and DNA methylation (n 13). [a]Full name of genes provided in Supplementary Table S6. **Abbreviations:** LC-MS, liquid chromatography-tandem mass spectrometry; MS-HRM, methylation sensitive-high resolution melting analysis; PBMC, peripheral blood mononuclear cells.

TABLE 6. Cohort study investigating the association between nutrients involved in one-carbon metabolism and DNA methylation (n 1). **Abbreviations:** LC-MS, liquid chromatography-tandem mass spectrometry; RBC, red blood cell

TABLE 1

PICOS criteria for inclusion and exclusion of studies

Parameter	**Criteria**
Participants	Adults aged 18 years and older
Intervention	Supplementation with B-vitamins or dietary intake of B-vitamins
Comparison	Supplementation with nutrients involved in one-carbon metabolism compared to placebo or control group in the case of randomized controlled trials, pre/post same group comparison for intervention studies
Outcomes	Outcomes of interest were changes in DNA methylation (global, gene-specific, genome-wide) in response to supplementation with folic acid and related B-vitamins and association between dietary intake of one-carbon metabolism nutrients and DNA methylation (global, gene-specific, genome-wide).
Study design	Randomized and non-randomized intervention studies. Observational studies including cross-sectional, case-control and cohort studies

TABLE 2

Randomized controlled trials investigating the effect of one-carbon metabolism related nutrient supplementation on DNA methylation (n 16)

Study	Population	Country	n	Intervention		Duration	DNA methylation analysis			Main findings related to DNA methylation
				FA µg/d	*Other µg/d or IU*	*Wk*	*Region*	*Technique*	*Tissue*	
Global DNA methylation										
Abratte 2009 [40]	Heathy	USA	45	235.3		12	Global	Cytosine extension assay	Mononuclear cells	No effect
Abratte 2009 [40]	Heathy	USA	45	470.6	344000 choline 122000 betaine	12	Global	Cytosine extension assay	Mononuclear cells	No effect
Abratte 2009 [40]	Heathy	USA	45	235.3	412000 choline 267000 betaine	12	Global	Cytosine extension assay	Mononuclear cells	No effect
Abratte 2009 [40]	Heathy	USA	45	470.6	486000 choline 349000 betaine	12	Global	Cytosine extension assay	Mononuclear cells	No effect
Crider 2011 [41]	Healthy	China	135	100	-	24	Global	LC-MS	Blood	No effect
Crider 2011 [41]	Healthy	China	135	400	-	24	Global	LC-MS	Blood	No effect
Crider 2011 [41]	Healthy	China	135	4000	-	24	Global	LC-MS	Blood	No effect
Fenech 1998 [23]	Healthy	Australia	63	700	7 B-12	12	Global	Methyl acceptance assay	Lymphocyte	No effect
Fenech 1998 [23]	Healthy	Australia	63	2000	20 B-12	12	Global	Methyl acceptance assay	Lymphocyte	No effect
Figueiredo 2009 [34]	Colorectal adenoma	USA	388	1000	-	156	Global	Pyrosequencing	Colon	No effect
Jung 2011 [24]	Hyper-homocysteine	The Netherlands	216	800	-	156	Global	LC-MS	Leukocyte	No effect

Kim 2001 [42]	Adenoma	USA	20	5000	-	52	Global	Methyl acceptance assay	Colon	↑Methylation (p = 0.02)
Nanayakkara 2008 [43]	Chronic kidney disease	The Netherlands	78	5000	1000 B-6 1000 B-12	52	Global	LC-MS	Leukocyte	No effect
O'Reilly 2016 [33]	Adenoma	Ireland	20	600	-	34	Global	Modified alkaline comet assay	Colon	↑Methylation (p < 0.001)
Pufulete 2005 [19]	Colorectal adenoma	UK	33	400	-	10	Global	Methyl acceptance assay	Leukocyte Colon	↑Methylation in colonic mucosa (p = 0.09) leukocytes (p = 0.05)
Pusceddu 2016 [31]	Elderly subjects	Germany	60	500	500 B-12 50000 B-6 1200 vit D 456000 Ca	12	Global	Pyrosequencing	Whole blood	↑Methylation
Stopper 2008 [32]	Hemodialysis	Germany	27	6428.6	-	20	Global	LC-MS/MS	Whole blood	No effect
Stopper 2008 [32]	Hemodialysis	Germany	27	6428.6	142.9 B-12	20	Global	LC-MS/MS	Whole blood	No effect
Gene-Specific Methylation										
Van den Donk 2007 [44]	Colorectal adenoma	The Netherlands	81	4600	1100 B-12	24	[a]*MGMT, MLH1, p14, p16, APC, RASSF1A*	GC-MS MSP	Colorectal	↑Methylation (OR = 1.67, p = 0.08)
Wallace 2010 [45]	Colorectal adenoma	USA & Canada	388	1000	-	156	*ESR1, SFRP1*	Pyrosequencing	Colorectal	No effect
Both Global and Gene-specific Methylation										
Al-Ghnaniem Abbadi 2013 [22]	Colorectal adenoma	UK	29	400	-	10	Global [a]*ESR1, MLH1*	Methyl acceptance assay	Colon	No effect global, *ESR1, MLH1*

								Pyrosequencing		
Obeid 2018[20]	Elderly subjects	Germany	63	500	500 B-12, 50000 B-6 1200 vit D 456000 Ca	52	Global [a]*ASPA, ITGA2B, PDE4C*	Pyrosequencing	Whole blood	↑LINE-1 methylation ↑ *ASPA* methylation (p = 0.046) ↑ *PDE4C* methylation (p = 0.062) No effect *ITGA2B*
Genome-wide DNA methylation										
Kok 2015 [21]	Elderly subjects	The Netherlands	87	400	500 B-12	104	Genome-wide	Illumina 450k array	Buffy Coat	Differential methylation at 162 positions upon FA/vB-12 supplementation (1 DMP, cg19380919 sig) in intervention compared to placebo 6 DMRs differed between intervention and placebo groups Serum folate and vitamin B-12 significantly related to DNA methylation of 173 and 425 regions respectively.

[a]Full name of genes provided in **Supplementary Table S6**

Abbreviations: GC-MS, gas chromatography-mass spectrometry, LC-MS, liquid chromatography-tandem mass spectrometry

TABLE 3

Intervention studies investigating the effect of nutrients involved in one-carbon metabolism on DNA methylation (n 10)

Study	Population	Country	n	Intervention		Duration	DNA methylation analysis			Main findings related to DNA methylation
				FA µg/d	*Other µg/d*	*Wk*	*Region*	*Technique*	*Tissue*	
Global DNA Methylation										
Abratte 2009 [40]	Healthy	USA	45	78.24	344000 choline 122000 betaine	2	Global	Cytosine extension assay	PBMC	No effect
Abratte 2009 [40]	Healthy	USA	45	235.3	344000 choline 122000 betaine	12	Global	Cytosine extension assay	PBMC	No effect
Abratte 2009 [40]	Healthy	USA	45	470.6	344000 choline 122000 betaine	12	Global	Cytosine extension assay	PBMC	No effect
Abratte 2009 [40]	Healthy	USA	45	235.3	412000 choline 267000 betaine	12	Global	Cytosine extension assay	PBMC	No effect
Abratte 2009 [40]	Healthy	USA	45	470.6	486000 choline 349000 betaine	12	Global	Cytosine extension assay	PBMC	No effect
Axume 2007 [46]	Healthy	USA	43	79.4	-	12	Global	Cytosine extension assay	PBMC	No effect
Axume 2007 [46]	Healthy	USA	43	235.3	-	7	Global	Cytosine extension assay	PBMC	↓Methylation *MTHFR* 677TT ($p < 0.05$)
Axume 2007 [46]	Healthy	USA	43	470.6	-	7	Global	Cytosine extension assay	PBMC	↓Methylation *MTHFR* 677TT ($p < 0.05$)
Ellingrod 2015 [47]	Schizophrenia	USA	35	5000	-	12	Global	LUMA	Whole blood	↑Methylation ($p < 0.0001$)
Hubner 2013 [48]	Healthy	Germany	34	500	500 B-12 50000 B-6 1200IU vit D, 456000 Ca	52	Global	Pyrosequencing	Whole blood	No effect at 3 sites ↑Methylation at CpG site 317 ($p = 0.044$)
Ingrosso	Uremia/	Italy	14		15000 MTHF	8	Global	Cytosine	PBMC	↓Methylation

2003 [49]	Hyper-homocysteine							extension assay		
Jacob 1998 [50]	Healthy	USA	10	56	-	5	Global	Methyl acceptance assay	Lymphocyte	↓Methylation
Jacob 1998 [50]	Healthy	USA	10	111	-	4	Global	Methyl acceptance assay	Lymphocyte	↑Methylation
Jacob 1998 [50]	Healthy	USA	10	286	-	3	Global	Methyl acceptance assay	Lymphocyte	↑Methylation
Jacob 1998 [50]	Healthy	USA	10	516	-	3	Global	Methyl acceptance assay	Lymphocyte	↑Methylation
Pizzolo 2011 [51]	Moderate hyper-homocysteine	Italy	7	5000	-	8	Global	LC/ESI-MS	PBMC	No effect
Rampersaud 2002 [53]	Healthy	USA	33	118	-	7	Global	Methyl acceptance assay	Leukocyte	↓Methylation ($p = 0.0025$)
Rampersaud 2002 [53]	Healthy	USA	33	200	-	7	Global	Methyl acceptance assay	Leukocyte	No effect
Rampersaud 2002 [53]	Healthy	USA	33	415	-	7	Global	Methyl acceptance assay	Leukocyte	No effect
Shelnutt 2004 [54]	Healthy	USA	41	67.6	-	7	Global	Methyl acceptance assay LC/ESI-MS	Leukocyte	↓Methylation ($p = 0.08$)
Shelnutt 2004 [54]	Healthy	USA	41	235	-	7	Global	Methyl acceptance assay LC/ESI-MS	Leukocyte	↑Methylation *MTHFR* 677TT ($p < 0.05$)
Both Global and Gene-Specific										
Protiva 2011 [52]	Healthy	USA	20	1000	-	8	Global Gene-specific	LC-MS Universal bead array	Colon	No effect

Abbreviations: LC/ESI-MS, liquid chromatography electrospray ionization mass spectrometry; LC-MS, liquid chromatography-tandem mass spectrometry, LUMA, luminometric assay; PBMC, peripheral blood mononuclear cells; MTHF, methyltetrahydrofolate

TABLE 4

Cross-sectional studies investigating the association between nutrients involved in one-carbon metabolism and DNA methylation (n 15)

Study	Population	Country	n	Nutrient Status		DNA methylation analysis			Main findings related to DNA methylation
				Biomarker	*Dietary*	*Region*	*Technique*	*Tissue*	
Global DNA Methylation									
Fenech 1998 [23]	Healthy	Australia	106	Folate, B-12	-	Global	Methyl acceptance assay	Lymphocyte	No correlation
Friso 2002 [82]	Valvular heart disease/ Healthy	Italy	292	Folate, B-12, B-6	-	Global	LC-MS	PBMC	Positive correlation with folate ($p < 0.01$), No correlation with B-12 status
Friso 2005 [58]	Healthy	Italy	198	Folate, B-12, B-6	-	Global	LC-MS	Lymphocyte	Positive correlation in *MTHFR* 1298 AA /677TT genotypes compared to the wild-type ($p = 0.001$)
Kok 2007 [62]	Healthy	The Netherlands	109	Folate, B-12, B-6, B2	-	Global	LC-MS	Blood	No correlation
Perng 2014 [63]	MESA study	USA	987	-	Folate, B-12, B-6, methionine	Global	Pyrosequencing	Leukocyte	Positive correlation with Alu No correlation with LINE-1
Pufulete 2005 [64]	Healthy	UK	68	Folate, B-12	Folate	Global	Methyl acceptance assay	Colon	Negative correlation serum folate ($r = -0.311$, $p = 0.01$), RBC folate ($r = -0.356$, $p = 0.003$), vitamin B-12 ($r = -0.218$, $p = 0.08$)
Stenvinkel 2007 [65]	Chronic kidney disease	Sweden	155	Folate, B-12	-	Global	LUMA	Leukocyte	No correlation

Stern 2000 [66]	Healthy	USA	19	Folate	-	Global	Methyl acceptance assay	Leukocyte	Positive correlation in *MTHFR* 677TT genotype (r= 0.738; p = 0.02)
Wernimont 2011 [67]	Normative ageing study	USA	621	Folate, B-12, B-6	-	Global	Pyrosequencing	Buffy coat	Correlation ($p \leq 0.05$)
Gene-specific DNA Methylation									
Beckett 2016 [55]	Retirement health & lifestyle study	Australia	80	Folate, B-12	-	[a]*CY2R1, VDR, CYP27B1, CY24A1*	Epitect II methylation enzyme	Peripheral blood cells	Positive correlation (*VDR)*
Bollati 2014 [13]	Obese/ overweight	Italy	165		Folate, B-12	[a]*CD14, Et-1, iNOS, HERV-w, TNFα*	Pyrosequencing	Buffy coat	Negative correlation *TNFα* (β =-0.339, p = 0.012)
Coppede 2014 [56]	Colorectal cancer	Italy	107	Folate, B-12	-	[a]*APC, MGMT, MLH1, RASSF1A, CDKN2A, p16*	Methylation sensitive-high resolution melting	Tumor tissue	Negative correlation *MLH1* (p = 0.05)
Hirsch 2008 [61]	Healthy	Chile	111	Folate, B-12	-	[a]*ec-SOD*	Bisulfite sequencing	Lymphocyte	No correlation
Both Global and Gene-specific DNA Methylation									
Geisel 2005 [59]	Healthy	Germany	71	Folate, B-12, B-6	-	Global [a]*p66SHc*	Pyrosequencing	Whole blood	No correlation
Hanks 2013 [60]	Healthy	UK	336	Folate, B-12	Folate	Global [a]*ESR1, MYOD1, IGF2, N33, APC, MLH1, MGMT*	Pyrosequencing	Colon	No correlation global Negative correlation *MGMT* (p = 0.001)

[a]Full name of genes provided in **Supplementary Table S6**

Abbreviations: LC-MS, liquid chromatography-tandem mass spectrometry; LUMA, Luminometric methylation assay, PBMC - peripheral blood mononuclear cells

TABLE 5

Case-control studies investigating the association between nutrients involved in one-carbon metabolism and DNA methylation (n 13)

Study	Population	Country	n	Nutrient status		DNA methylation			Main findings related to DNA methylation
				Biomarker	*Dietary*	*Region*	*Technique*	*Tissue*	
Global DNA Methylation									
Badiga 2016 [69]	Cervical intraepithelial neoplasia	USA	132 case 325 control	Folate, B-12	-	Global	Pyrosequencing	PBMC	Negative correlation
Bednarska-Makaruk 2016 [70]	Dementia	Poland	102 case 45 control	Folate, B-12, 5-MTHF	-	Global	Imprint methylated kit	Leukocyte	Positive correlation (p = 0.013)
Friso 2013 [71]	Cancer	Italy	68 cancer 68 control	Folate	-	Global	LC-MS	PBMC	Positive correlation in *MTHFR* 677TT genotype
Nan 2013 [73]	Colorectal cancer	USA	358 CRC 661 control	Folate, B-12, B-6	Folate	Global	LC-MS	Leukocyte	No correlation
Pufulete 2003 [74]	Colorectal adenoma/ cancer	UK	63 adenoma 76 control	Folate, B-12	Folate	Global	Methyl acceptance assay	Leukocyte/ colon	Negative correlation Serum folate (r = -0.243, p = 0.009), RBC folate (r =-0.282, p = 0.002), folate status score (r = -0.295, p=0.001) in colon tissue No correlation in leukocytes
Tremolizzo 2013 [76]	ALS	Italy	96 ALS 87 control	Methionine	-	Global	Methyl acceptance assay	Whole blood	Positive correlation (r = 0.216, p = 0.043)
Wang 2012 [78]	Chromate exposure	China	115 case 60 control	Folate	-	Global	ELISA kit	Whole blood	Positive correlation (r = 0.163, p = 0.032)
Gene-Specific DNA Methylation									
Kim 2011 [72]	Colorectal cancer	Korea	67 CRC 53 control	Folate	-	[a]*p16, p73, MLH1*	Methylation-specific PCR	White blood cells	Positive correlation *p73*

Tannorella 2015 [75]	Alzheimer's disease	Italy	120AD 115control	Folate, B-12	-	[a]*PSEN1, BACE1, MTHFR, DNMT1, DNMT3A DNMT3B, MTHFR*	Methylation MS-HRM	Peripheral blood	Positive correlation *MTHFR* methylation (r = 0.21; p = 0.002)
Van Guelpen 2009 [77]	Colorectal adenocarcinoma	Sweden		Folate, B-12	-	[a]*CDKN2A, MLH1, IGF2, CACNA1G, NEUROG1, RUNX3, SOCS, CRABP1*	MethylLight real time PCR	Colon	Positive correlation vitamin B-12 *CACNA1G* (p = 0.047), folate *RUNX3* (p = 0.038)
Wei 2015 [79]	Ischemic stroke	Malaysia	297case 110 control	Folate, B-12	-	[a]*MTHFR*	Pyrosequencing	Whole blood	Positive correlation Serum folate (r = 0.106, p = 0.032), vitamin B-12 (r = 0.114, p = 0.022)
Zhang 2014 [80]	Essential hypertension	China	258 EH 137 control	Folate, B-12	-	[a]*TERT*	Methylation-specific PCR	Leukocyte	No correlation
Both Global and Gene-Specific DNA Methylation									
Al-Ghnaniem 2007 [68]	Colorectal neoplasia	UK	156 case 76 control	Folate, B-12	-	Global [a]*ESR1, MLH1*	Methyl acceptance assay Pyrosequencing	Colon	Inverse association between serum folate/vitamin B12 and global methylation in adenoma patients. Negative correlation *ESR1* (r = 0.239, p = 0.003)

[a]Full name of genes provided in **Supplementary Table S6**

Abbreviations: LC-MS, liquid chromatography-tandem mass spectrometry; MS-HRM, methylation sensitive-high resolution melting analysis; PBMC, peripheral blood mononuclear cells

TABLE 6

Cohort study investigating the association between nutrients involved in one-carbon metabolism and DNA methylation (n 1)

Study	**Population**	**Country**	**n**	**Nutrient Status**		**DNA methylation**			**Main findings related to DNA methylation**
				Biomarker	*Dietary*	*Region*	*Technique*	*Tissue*	
Global DNA Methylation									
Bae 2014 [81]	Postmenopausal women	USA	408	Folate, B-12, B-6, choline, betaine	Folate, B-12, B-6, B2	Global	LC-MS	Leukocyte	Positive correlation Plasma folate (r = 0.20, p = 0.04) , RBC folate (r = 0.24, p = 0.01), vitamin B-12 (r = 0.18, p = 0.06)

Abbreviations: LC-MS, liquid chromatography-tandem mass spectrometry; RBC, red blood cell